Relation Between Macronuclear DNA and Total Protein Content and Generation Time in the *Chilodonella steini* (*Ciliata*) Sister Cells

Summary

Using the monotone dependence function (mdf) together with correlation coefficient it was found that the Ma-DNA content as well as total protein content are regularly, linearly, positively and strongly dependent in sister cells (proter-opisthe) of *Chilodonella steini*. Additionally it was shown that proter-opisthe ordering is irrelevant to Ma-DNA and protein contents.

Analysis of sister cell generation times (TG) confirmed the existence of regular, linear, positive and strong codependence.

The relations between Ma-DNA and total protein contents, between protein content and TG, and between Ma-DNA content and TG were also described. There is a weak, linear dependence between Ma-DNA and total protein contents. Relations of TG and Ma-DNA content or TG and total protein content are non-linear and not even monotone. Low and high levels of DNA or proteins are connected with long generation times.

Keywords: Correlation coefficient; Ma-DNA content; Generation times; Monotone dependence function; Protein content; Sister cells.

1. Introduction

Contrary to the majority of *Eucariota* in *Ciliata* macronuclear DNA contents (Ma-DNA) varies very greatly depending on conditions, developmental stages and clone age. After division, newly-formed cells almost always differ from each other in respect to chromatin content (Raikov 1982).

The distribution of cell content during cytokinesis is not a precise process in all animal cells. The cleavage furrow divides cytoplasm and its organelles into more or less equal parts, hence cells formed after division differ in their mass (Kimball *et al.* 1971) and composition of certain organelles (Shepard 1965). Despite this, both, the mean levels and the ranges of variation in protein content are similar in cell lines maintained for many generations. This would suggest the existence of a controlling equalizing mechanism, ensuring relative constancy of these characters in successive generations. A knowledge of the generation time (TG) relation to Ma-DNA and total protein contents seems to be important for analysis of the complex mechanisms controlling the cell cycle.

The unequal distribution of genetic and cytoplasmic material between sister cells observed in ciliates provides the possibility of defining the effect of such inequalities on cell cycle duration. In some species, *e.g.*, *Chilodonella steini,* it is easy to distinguish between proters and opisthes, which would make it possible to ascertain whether distribution of these components and shorter or longer TG depend on the kind of cell in the pair.

Thus the aim of this study was to estimate:

1. the relations of Ma-DNA levels as well as the total protein contents between proters and opisthes,
2. the relation of TG duration between sister cells, and its stability in three successive generations,
3. the relation between Ma-DNA levels and total protein contents,
4. the relation between TG duration and Ma-DNA levels and total protein contents.

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When cell wall fractions were sonicated, a Model W-225 R Sonicator (Heat Systems-Ultrasonics, Inc.[2] was used. The sample tube was bathed in ice water and sonicated with a standard microtip at a 50% pulsed cycle for 5 minutes.

2.3. Biochemical Assays

β-glucosidase activity (β-G) was measured with 5 mM PNP-β-glucose in 0.1 M sodium citrate buffer at pH 5.5. Assays were carried out in 1 ml volume and 10 to 100 µl of enzyme (25 µg protein as maximum) was added to initiate the reaction. Assay tubes were incubated for 2 to 10 minutes at 38 °C in a shaking water bath and the reaction was stopped by the addition of 2.5 ml of 0.2 M sodium carbonate. All assays were initiated, terminated, and read at timed intervals. Absorbance was read at 405 nm and a standard curve was determined with 1 mM PNP. β-G activity in crude homogenates was linear, with respect to time and protein concentration, under these assay conditions.

Acid phosphatase activity (AP) was measured with 2.5 mM PNP-phosphate (0.1 M sodium citrate buffer pH 4.5) at 38 °C for 10 to 30 minutes. Malate dehydrogenase activity (MDH) was assayed according to TING (1968) and triose phosphate isomerase activity (TIM) was determined by the coupled reaction of SCHNARRENBERGER *et al.* (1972). Cytochrome c oxidase (CCO) was measured as described by HODGES and LEONARD (1974) except Triton X-100 was used in place of digitonin. To determine enzyme activities in cell wall preparations, it was necessary to either centrifuge the reaction tube or filter off the cell walls before reading the absorbance.

Protein was estimated by a modification of the Lowry procedure (MARKWELL *et al.* 1978) and when cell wall fractions were analyzed, samples were solubilized in 0.1 N NaOH for 20 hours at 4 °C prior to protein analysis. Phospholipids were quantitated by analysis of phosphatidyl choline (DITTMER and WELLS 1969) which represented the most abundant phospholipid in membranes.

2.4. Electron Microscopy

Isolated particulate fractions were suspended in 2.5% glutaraldehyde in pH 7.0 cacodylate buffer. The fixed particulate suspensions were then either pelleted at 84,000 g in an SW 28.1 rotor, or collected on 0.2 micron millipore filters and post-fixed in 1% OsO_4. After several buffer rinses, the fixed samples were dehydrated in a graded series of acetone, embedded in Spurr's epoxy resin, cured, and thin-sectioned for electron microscopy. Sections were post-stained with uranyl acetate and lead citrate and viewed with a Zeiss EM 10 B electron microscope.

3. Results and Discussion

In this study, small cell wall fragments initially filtered through cheesecloth were further purified (NAKAGAWA *et al.* 1971). These cell wall fragments were representative of the total cortical cell wall population (data not shown) and were used because they could be pipeted conventionally and were easier to handle during enzyme assays. In addition, these fragments could be firmly pelleted by low speed centrifugation and this minimized any losses during washing experiments.

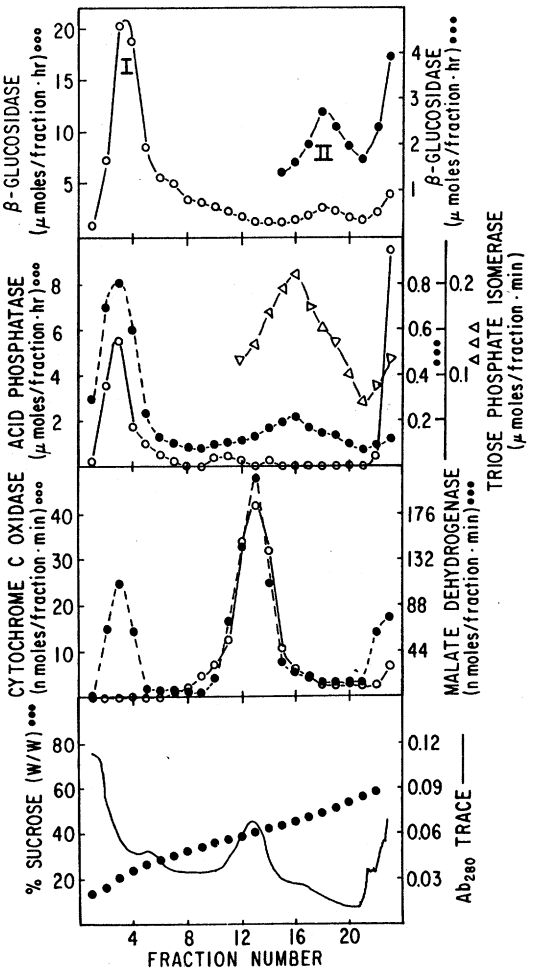

Fig. 1. Linear sucrose density centrifugation of a crude cell wall fraction isolated from corn roots. The 1,000 g pellet was overlaid on a 20 to 60% linear sucrose (w/w) gradient (17 ml), centrifuged at 84,000 g for 15 hours, and fractionated into 0.75 ml fractions. The cell wall pellet at the bottom of the gradient was suspended in buffer and included as fraction 23. Portions of the gradient containing triose phosphate isomerase and β-glucosidase activity were plotted on two scales

3.1. Linear Sucrose Density Gradient Centrifugation of a Crude Cell Wall Fraction

The crude cell wall fraction (1,000 g pellet) was overlaid on a 20 to 60% (w/w) sucrose gradient and centrifuged for 15 hours at 84,000 g to ensure that membranes and intact organelles reached equilibrium densities (NAGAHASHI 1985). This long term centrifugation was only necessary to assess the types of contaminants present in the crude overlay. The gradient pellet was

[2] Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

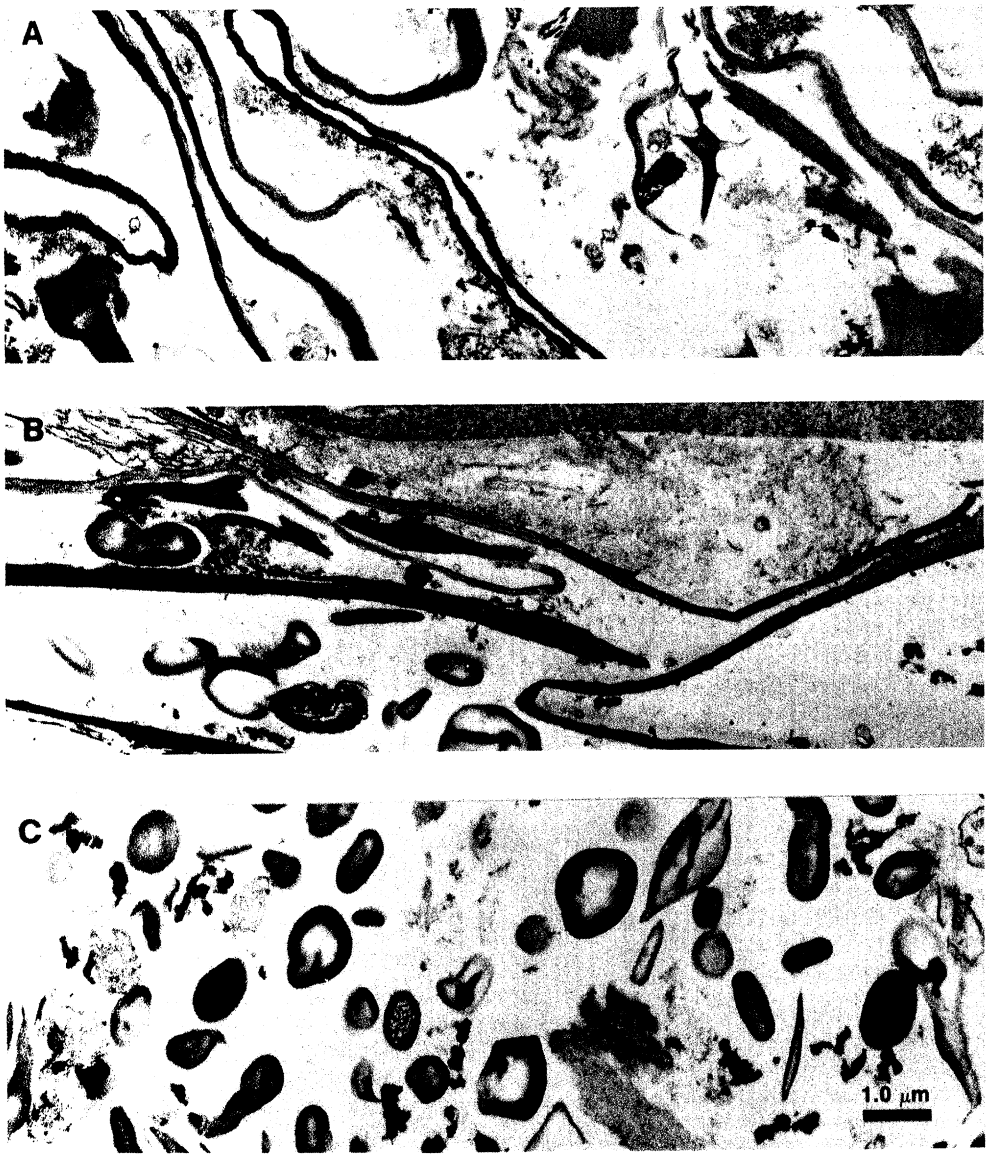

Fig. 2. Electron micrographs of the cell wall fraction which pelleted through the sucrose gradient shown in Fig. 1. The outer edge of the pellet was analyzed at the top (*A*), middle (*B*), and bottom (*C*)

expected to contain the cell walls since an earlier report indicated that walls pelleted through a 20 ml cushion of 48% sucrose after centrifugation for 30 minutes at 58,750 g (LAI and THOMPSON 1970). Marker enzymes for organelles most likely to be present in the crude overlay were monitored. In addition, β-G and AP were assayed since previous reports indicated these enzymes were bound to cell walls (HUBER and NEVINS 1981, PIERROT and VAN WIELINK 1977, STEPHENS and WOOD 1974, YUNG and NORTHCOTE 1975).

Intact proplastids equilibrated at 45% sucrose (TIM activity) while mitochondria (CCO activity) were found at 41% (Fig. 1). The MDH activity at 41% sucrose was due to mitochondria and possibly microbodies since these organelles have the same density under the centrifugation conditions employed (NAGAHASHI and BAKER 1984). The 280 nm trace showed that these organelles were a major source of protein contamination in the crude cell wall fraction (Fig. 1). The absorbance trace also indicated a considerable amount of soluble compounds (phenols, nucleic acids, and proteins) were removed by the sucrose gradient and these compounds remained near the top after centrifugation. All enzyme activities in this region of the gradient (MDH, TIM, AP, β-G) were soluble and not membrane-bound. This was demonstrated by the fact

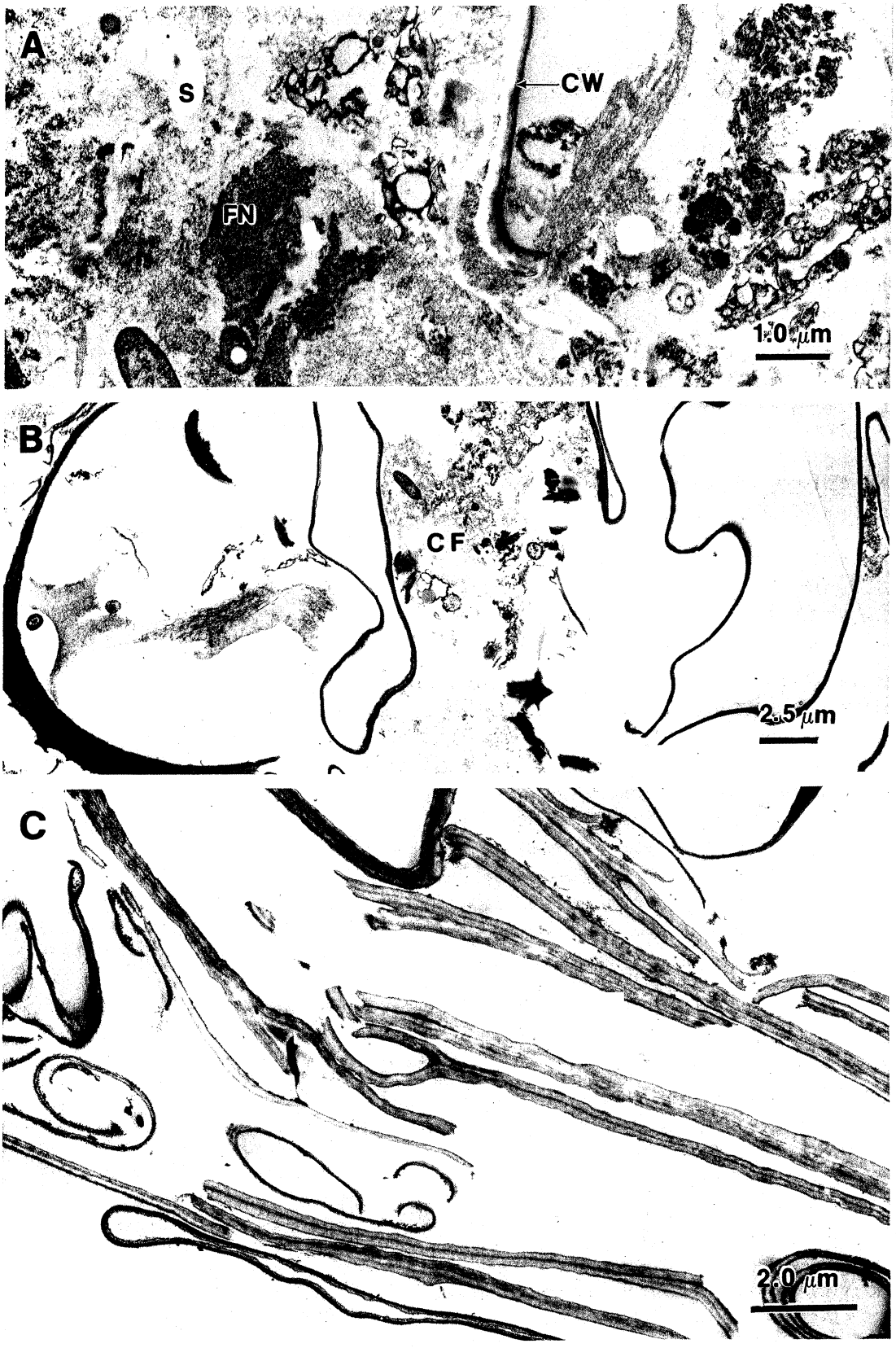

Fig. 3. Electron micrographs of cell wall fractions isolated from corn roots at various stages of purification. *A* Representative micrograph of the center of a crude cell wall fraction (1,000 g pellet). The crude cell wall pellet was stratified in similar fashion to Fig. 2. *B* Representative micrograph of the mid-center region of the cell wall pellet isolated after sucrose gradient centrifugation (see Fig. 2 for other areas of the pellet). *C* Micrograph of purified cell walls which were prepared for electron microscopy by collecting fixed material on 0.2 micron filters. Trapping of cell walls on filters eliminated stratification problems observed in Fig. 2 and facilitated the ultrastructural analysis. *S* starch granule, *FN* fragmented nucleus, *CW* cell wall, *CF* cytoplasmic fragment

that they did not reach equilibrium density after centrifugation for 15 hours (data not shown) but continued to move further into the gradient after prolonged centrifugation (NAGAHASHI and BAKER 1984). Unexpectedly, 80% of the total β-G activity initially associated with the crude overlay was recovered as a soluble enzyme near the top of the gradient (Peak I). Equally surprising was the presence of a particulate form of β-G (Peak II) which equilibrated at 51% sucrose (Fig. 1). The source of Peak II was not identified; however, the enzyme activity did not coincide with any organelle marker. Electron micrographs of this region of the gradient showed mainly cytoplasmic fragments and unidentified membranes (data not shown). Recent work has shown that Peak I was the soluble form of Peak II activity and was not cell wall in origin (NAGAHASHI *et al.* 1985).

3.2. Analysis of the Cell Wall Fraction Pelleting Through the Linear Gradient

When the gradient pellet was suspended and included as a gradient fraction (Fig. 1, fraction 23), all enzymes monitored were still detected. β-G and AP activity were expected; however, the presence of MDH, CCO, and TIM activity suggested that the cell walls still contained entrapped contaminants. This was confirmed by ultrastructural examination with the electron microscope. The major contaminants of the cell wall fraction were starch bodies and cytoplasmic fragments although membranous components were also observed (Fig. 2). The gradient pellet was stratified in a peculiar manner (Fig. 2). Starch granules formed an annulus near the outer edge of the pellet and when this area of the pellet was examined from top to bottom, starch granules were primarily found in the bottom half (Fig. 2). This stratification was not reported in previous ultrastructural examinations of isolated cell walls which showed a very limited portion of the purified cell wall fraction (BACIC and STONE 1981, GORDON *et al.* 1977, KING and BAYLEY 1965). When the center of the pellet was examined from top to bottom, cell walls were dispersed throughout (Fig. 3 *B*).

No biochemical markers were used to detect nuclei; however, intact nuclei were not observed morphologically in the crude overlay (Fig. 3 *A*) or in the gradient pellet (Figs. 2 and 3 *B*). It was likely that the nuclei were either ruptured during the homogenization procedure (JACOBSEN 1968) or broken when the 1,000 g fraction was pelleted or resuspended (TAUTVYDAS 1971) as suggested by Fig. 3 *A*.

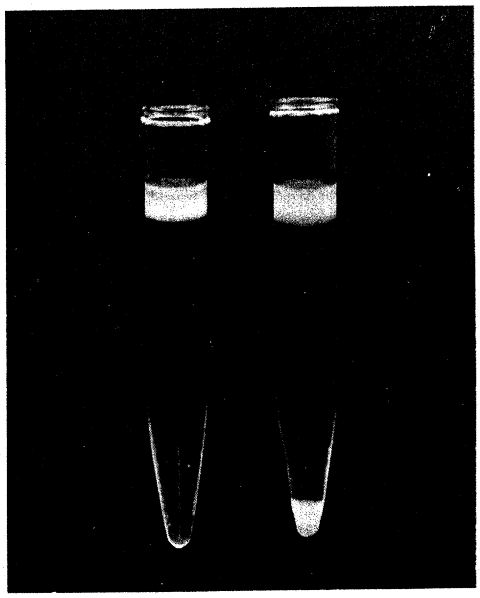

Fig. 4. Separation of starch bodies from cell walls of corn roots by gravity sedimentation through a 15% (w/w) sucrose cushion. Cell walls were suspended in 30 mM HEPES-MES pH 7.5, sonicated for 5 minutes with a 50% pulsed cycle in an ice bath, overlaid on the sucrose cushion, and placed in a refrigerator for one 1½ hours to sediment the cell walls. Starch granules and cytoplasmic contaminants remained on top. Left, time 0. Right, after 80 minutes

To keep nuclear membranes from pelleting with the cell walls, the linear gradient composition of 20 to 60% sucrose was used to insure that these membranes stayed suspended in the gradient after centrifugation. The only other report which used sucrose gradients to purify cell walls chose a gradient design (20 to 40% linear gradient) and centrifugation conditions (2,000 g for 12 minutes) which kept tomato pericarp walls in suspension near the bottom of the gradient (NAKAGAWA *et al.* 1971). Because cell walls suspended in gradients were more difficult to collect and were likely to have greater cross contamination by membranes and organelles, a different gradient design and centrifugation conditions were chosen. The 20 to 60% gradient only had to be centrifuged at 84,000 g (minimum g force was not determined) for 15 minutes to pellet cell walls. The sucrose gradient which contained intracellular contaminants was simply decanted from the pellet.

3.3. Further Purification of Cell Walls by Sonication and Removal of Starch Granules

The gradient pellet was suspended in 30 mM HEPES-MES pH 7.5 plus 1 mM β-SH and sonicated for 5 minutes in order to remove tightly adhering cytoplasm. The sonicated suspension was overlaid on a 15% sucrose (w/w) cushion (13 ml) and placed in a

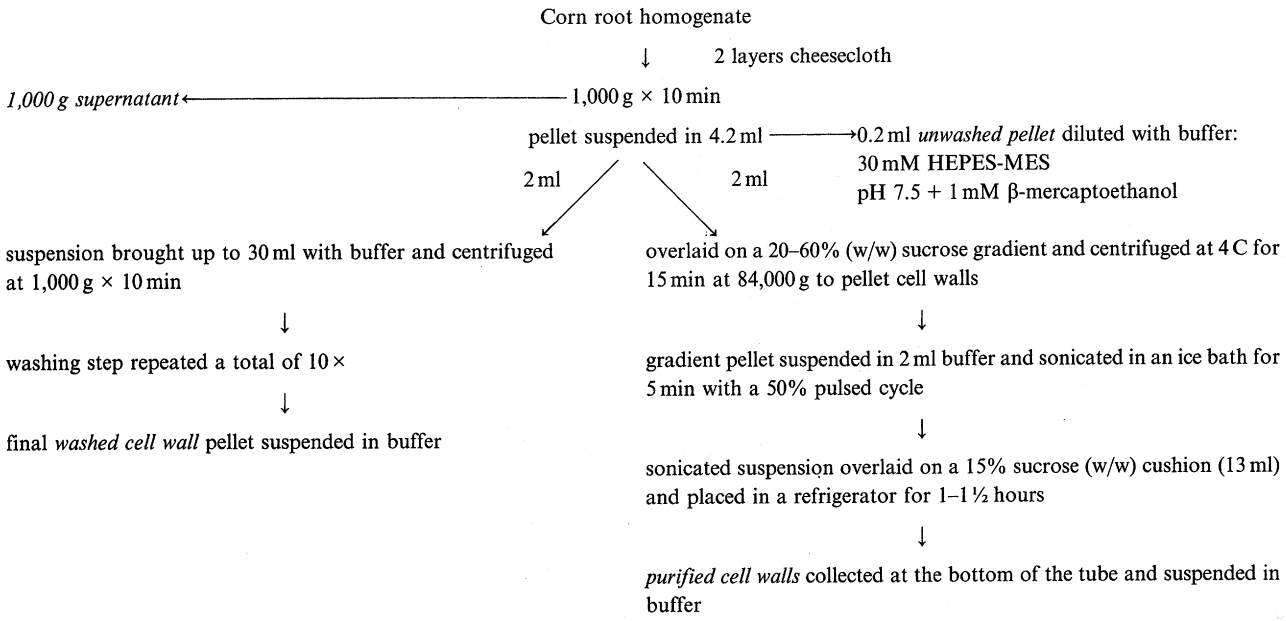

Fig. 5. Flow diagram of two different cell wall purification procedures used in this study

refrigerator for 1 to 1½ hours (approximately 1 g force). After this time, starch granules and residual cytoplasmic contaminants remained on top of the cushion while the cell walls sedimented to the bottom (Fig. 4). Ultrastructural examination showed these cell walls were very pure (Fig. 3 *C*). To make the analysis considerably easier, cell walls were collected on a 0.2 micron millipore filter to avoid the stratification problems encountered in pelleted fractions.

For certain plant tissues, it would be advantageous to use this gravity sedimentation technique for starch removal compared to DMSO or α-amylase treatment (HARRIS 1983). However, separation was not achieved with storage tissues such as potato tubers which contained large starch grains. Regardless of tissue type, to study cell wall proteins requires the removal of starch grains because they represent a source of protein (PERDON *et al.* 1975).

3.4. Comparison of Cell Wall Isolation Procedures

To directly compare our purification procedure with the commonly used multiple washing procedure, cell walls were isolated and purified as shown in Fig. 5. Walls purified by washing 10 times contained low but detectable levels of PC, CCO, MDH, and TIM activity. Under the electron microscope, cytoplasmic fragments and some membrane contaminants were still observed (micrograph not shown).

In comparison, cell walls isolated by the new procedure did not contain CCO, MDH, or TIM activity (Tab. 1). The absence of these markers was confirmed by ultrastructural examination which showed only cell walls (results identical to Fig. 3 *C*). PC could only be detected when one-half of the total purified cell wall fraction was analyzed. Since no organelle membranes were present and the level of phospholipid was low (0.2% of the total homogenate), it was likely that the PC detected was due to plasmodesmata. The presence of plasmodesmata in purified cell walls was reported earlier (BACIC and STONE 1981).

The association of MDH with cell walls has been controversial (GROSS 1977, STEPHENS and WOOD 1974, YUNG and NORTHCOTE 1975) although two reports have used this activity as an intracellular marker for cytoplasmic contamination (DRACUP *et al.* 1984, STRAND *et al.* 1976). MDH activity at the bottom of the sucrose gradient in Fig. 1 and activity associated with cell walls after multiple washing (Tab. 1) were the result of cytoplasmic contamination since purified corn root cell walls did not contain MDH activity (Fig. 3 *C* and Tab. 1).

AP and β-G were associated with cell walls purified by both purification procedures (Tab. 1) but the specific activities were higher in the cell walls purified by the new procedure (AP = 21.2 μmoles/mg · hour, β-G = 20.0 μmoles/mg · hour) compared to the con-

Table 1. *Comparison of the distribution of various biochemical markers in cell walls purified by the two different methods given in Fig. 5*

Fraction	Total amounts					
	nmoles	μmoles/minute · fraction			μmoles/hour · fraction	
	PC	CCO	MDH	TIM	AP	β-G
1,000 g supernatant	4,964	10.38	126.60	303.48	6,825.7	2,536
1,000 g pellet (unwashed)	325.8	0.56	4.09	18.68	139.3	634.1
1,000 g pellet (washed 10×)	29.7	0.07	0.33	1.94	16.7	17.4
Sucrose gradient-sonicated-gravity sedimented cell walls	12.2	0	0	0	15.9	15.0

PC, phosphatidyl choline; CCO, cytochrome c oxidase; MDH, malate dehydrogenase; TIM, triose phosphate isomerase; AP, acid phosphatase; β-G, β-glucosidase.

ventional method (AP = 16.4 μmoles/mg · hour, β-G = 17.2 μmoles/mg · hour). Although these enzymes were found in low amounts in the purified cell walls (Tab. 1), they were tightly bound and confirmed the low amount of activity previously reported in the *in vivo* localization studies on corn roots (ASHFORD 1970, ASHFORD and MCCULLY 1970).

For any cell wall purification procedure, it is necessary to determine the criteria by which the purity of isolated cell walls can be judged. It is recommended that a membrane marker (CCO or PC for example) and a soluble cytoplasmic marker (such as TIM) be used in the negative mode (QUAIL 1979) to monitor their removal during purification. In conjunction with the use of biochemical markers, the purified cell walls should be examined at the ultrastructural level since membrane vesicles or fragments and cytoplasmic fragments cannot be detected with a light microscope. If a unique, readily assayable marker for cell walls can be found (HARRIS 1983), then this marker could be used in the positive mode which would allow a direct measure of enrichment and yield or recovery of purified cell walls.

4. Conclusions

In a recent report (LI *et al.* 1983), the cell walls isolated from lily pollen and roots had to be washed 27 times to ensure removal of adhering cytoplasm and membranes. Our results confirmed that resuspension-resedimentation only slowly removed these contaminants (data not shown for the intermediate washing steps in Tab. 1). Cytoplasmic fragments and starch bodies were the most difficult contaminants to remove by the conventional centrifugation procedure. Rapid centrifugation of the crude cell wall fraction through a sucrose gradient removed a considerable amount of membranes, organelles, and soluble protein contaminants (Fig. 1). Sucrose gradient centrifugation provided a distinct advantage over buffer-H_2O washing since the density of the gradient prevented large membrane vesicles or large organelles from pelleting with the cell walls. Sonication of the sucrose gradient pellet followed by gravity sedimentation through a 15% sucrose cushion removed the starch bodies and remaining cytoplasmic contaminants. The sucrose gradient-sonication-gravity sedimentation technique provides a rapid way to purify cell wall fragments and can be used in place of the commonly used, less efficient, multiple washing procedure. Because the yield of cell wall fragments was low, the technique developed may be most useful as an analytical method particularly suited for determining the types of contaminants in cell wall fractions and for screening potential cell wall marker enzymes.

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References

ASHFORD, A. E., 1970: Histochemical localization of β-glycosidases in roots of *Zea mays*. II. Changes in localization and activity of β-glucosidase in the main root apex. Protoplasma **71**, 389–402.

— MCCULLY, M. E., 1970: Localization of naphthol AS-BI phosphatase activity in lateral and main root meristems of pea and corn. Protoplasma **70**, 441–456.

BACIC, A., STONE, B. A., 1981: Chemistry and organization of aleurone cell wall components from wheat and barley. Aust. J. Plant Physiol. **8**, 475–495.

DITTMER, J. C., WELLS, A. W., 1969: Quantitative and qualitative analysis of lipids and lipid components, p. 482. (Methods in Enzymology, Vol. 14.) New York: Academic Press.

DRACUP, M. N. H., BARRETT-LENNARD, E. G., GREENWAY, H., ROBSON, A. D., 1984: Effect of phosphorus deficiency on phosphatase activity of cell walls from roots of subterranean clover. J. exp. Bot. **35**, 466–480.

GORDON, A. H., HAY, A. J., DINSDALE, D., BACON, J. S. D., 1977: Carbohydr. Res. **57**, 235–248.

GROSS, G. G., 1977: Cell wall-bound malate dehydrogenase from horseradish. Phytochemistry **16**, 319–321.

HARRIS, P. J., 1983: Cell walls, A 25 (Isolation of membranes and organelles from plant cells; HALL, J. L., MOORE, A. L., eds.). New York: Academic Press.

HODGES, T., LEONARD, R. T., 1974: Purification of plasma membrane-bound adenosine triphosphatase from plant roots, p. 392. (Methods in Enzymology, Vol. 32.) New York: Academic Press.

HUBER, D. J., NEVINS, D. J., 1981: Partial purification of endo- and exo-β-D-glucanase enzymes from *Zea mays* L. seedlings and their involvement in cell-wall autohydrolysis. Planta **151**, 206–214.

JACOBSON, A. B., 1968: A procedure for isolation of proplastids from etiolated maize leaves. J. Cell Biol. **38**, 238–244.

KING, N. J., BAYLEY, S. T., 1965: A preliminary analysis of the proteins of the primary walls of some plant cells. J. exp. Bot. **16**, 294–303.

LAI, Y. F., THOMPSON, J. E., 1970: 5′-nucleotidase and glucose-6-phosphatase in a purified cell-wall fraction from *Phaseolus vulgaris*. Phytochem. **9**, 1017–1021.

LI, YI-QIN, CROES, A. F., LINSKENS, H. F., 1983: Cell-wall proteins in pollen and roots of *Lilium longiflorum:* Extraction and partial characterization. Planta **158**, 422–427.

MARKWELL, M. A. K., HAAS, S. M., BIEBER, L. L., TOLBERT, N. E., 1978: A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal. Biochem. **87**, 206–210.

NAGAHASHI, G., 1985: The marker concept in cell fractionation. (Modern Methods of Plant Analysis, New Series, Vol. I.) Berlin-Heidelberg-New York-Tokyo: Springer. In press.

— BAKER, A. F., 1984: β-glucosidase activity in corn root homogenates: Problems in subcellular localization. Plant Physiol. **76**, 861–864.

— SEIBLES, T. S., TU, S.-I., 1985: The pH dependent distribution of β-glucosidase activity in isolated particulate fractions. Plant Sci. Letts. **38**, 173–178.

NAKAGAWA, H., SEKIGUCHI, K., OGURA, N., TAKEHANA, H., 1971: Binding of tomato pectinesterase and β-fructofuranosidase to tomato cell wall. Agr. Biol. Chem. **35**, 301–307.

NARI, J., NOAT, G., RICARD, J., FRANCHINI, E., SAUVE, P., 1982: Purification and molecular properties of a cell-wall β-glucosyltransferase from soybean cells cultured *in vitro*. Plant Sci. Letts. **28**, 307–312.

PERDON, A. A., DEL ROSARIO, E. J., JULIANO, B. O., 1975: Solubilization of starch synthetase bound to *Oryza sativa* starch granules. Phytochem. **14**, 949–951.

PIERROT, H., VAN WIELINK, J. E., 1977: Localization of glycosidase in the wall of living cells from cultured *Convolvulus arvensis* tissue. Planta **137**, 235–242.

QUAIL, P., 1979: Plant cell fractionation. Ann. Rev. Plant Physiol. **30**, 425–484.

SCHNARRENBERGER, C., OESER, A., TOLBERT, N. E., 1972: Isolation of plastids from sunflower cotyledons during germination. Plant Physiol. **50**, 55–59.

SELVENDRAN, R. R., 1975: Analysis of cell wall material from plant tissues: Extraction and purification. Phytochem. **14**, 1011–1017.

STEPHENS, G. J., WOOD, R. K. S., 1974: Release of enzymes from cell walls by an endopectate-trans-eliminase. Nature **251**, 358.

STRAND, L. L., RECHTORIS, C., MUSSELL, H., 1976: Polygalacturonases release cell-wall-bound protein. Plant Physiol. **58**, 722–725.

TAUTVYDAS, K. J., 1971: Mass isolation of pea nuclei. Plant Physiol. **47**, 499–503.

TING, I. P., 1968: Malic dehydrogenase in corn root tips. Arch. Biochem. Biophys. **126**, 1–7.

YUNG, K.-H., NORTHCOTE, D. H., 1975: Some enzymes present in the walls of mesophyll cells of tobacco leaves. Biochem. J. **151**, 141–144.